Reference: *Biol. Bull.* **184:** 186–202. (April, 1993)

Control of Hatching in an Estuarine Terrestrial Crab. II. Exchange of a Cluster of Embryos Between Two Females

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Abstract. The eggs of an estuarine terrestrial crab, *Sesarma haematocheir* (akate-gani), are incubated by the female for about one month. In estuarine crabs larval hatching is synchronized with the nocturnal high tide. To investigate whether the female or the embryo controls the actual timing of the hatching, one cluster of embryos was detached from each of two ovigerous females and reciprocally transplanted. Hatching of the transplanted embryos was divided into the following three patterns according to the number of nights until either (or both) of the females released their larvae. In *Pattern I*, the transplanted clusters both hatched on the same night that the donor females released their larvae. In *Pattern II*, the hatching of one of the transplanted clusters was not controlled by the host female, whereas hatching of the other transplanted cluster was obviously induced. Finally, in *Pattern III*, not only the induction of hatching, but also the time of hatching, was controlled by the female. Hatching profiles of transplanted embryos transferred to aerated conditions indicated that hatching requires *three nights*, and that each embryo also has an endogenous rhythm for hatching. The female seems to play two roles in hatching: *i.e.*, initiation of the hatching process, and enhancement of hatching synchrony in each embryo. A plausible hypothesis explaining the mechanism of induction and the synchronization of hatching is presented.

Introduction

Clearly demarcated rhythmicities are often observed in the reproductive behaviors of both marine and terrestrial animals. A persistent question in reproductive rhythm research is whether the female or the embryo controls the actual timing of these behaviors. Rhythms of spawning (*i.e.*, shedding of gametes or fertilized eggs) or oviposition following gametogenesis must be controlled by the female alone. Examples of this phenomenon are the circadian rhythm of oviposition in the pink bollworm *Pectinophora gossypiella* (Pittendrigh and Minis, 1971), egg laying in the teleost *Oryzias latipes* (Egami, 1954; Ueda and Oishi, 1982), and the daily, tidal and lunar rhythms of spawning in many kinds of marine invertebrates (Korringa, 1947; Pearse, 1990).

Embryonic development proceeds within the eggs oviposited by the female, and hatching occurs after a certain period. A circadian rhythm of hatching appears in the bollworm *P. gossypiella*. Eggs of this species, maintained at 20°C, hatched 10–13 days after they were oviposited; the eggs were transferred from constant light (LL) to constant darkness (DD) every 5.5 h during embryonic development, and hatching was monitored (Minis and Pittendrigh, 1968). This experiment suggested that a circadian pacemaker controlling hatching is differentiated at least around the midpoint of embryogenesis, *i.e.*, 6–7 days after oviposition.

Obvious rhythmic patterns are also observed in the hatching of marine crustaceans (Sastry, 1983; DeCoursey, 1983). But eggs of most marine and freshwater crustaceans are incubated by the female until hatching occurs. This phenomenon complicates the control of the larval hatching rhythm. Indeed, the timing of larval hatching is synchronized with day-night, tidal, and lunar cycles, but whether it is the female or the embryo that controls the actual timing of hatching remains unclear.

This question has only been investigated with respect to eggs already detached from the female (Saigusa, 1992c), the role of the female is still unknown. This paper focuses on the female control of larval hatching in an estuarine

Received 28 May 1991; accepted 25 January 1993.

terrestrial crab, *Sesarma haematocheir*, and reports on an embryonic exchange method that was used in the investigation. Eggs of this species consist of eight clusters. Two of such clusters, one from each of two ovigerous females, were detached and exchanged by reciprocal transplantation. The transplanted eggs survived on the host females, and most of them successfully hatched. Hatching in the transplanted eggs was clearly divided into *three patterns* depending upon the number of nights intervening between the exchange and the occurrence of hatching in both or either of the females.

These results suggest that the female triggers the hatching process in each embryo, but that each embryo has also an endogenous rhythm of hatching. In response to some (unknown) stimuli released from the female, each embryo must initiate its hatching process around the time of nocturnal high tides, and hatching occurs 48–49.5 h later. Since all of the female-attached embryos hatch within a very short time, the female should have some mechanism for enhancing hatching synchrony just before the larval release.

This paper provides evidence that the control of hatching involves cooperation between female and embryo. Based on the data reported here, I present a hypothesis that explains the mechanism controlling the daily timing of larval hatching in *Sesarma haematocheir*.

Materials and Methods

Maintenance of crabs and monitoring of larval release in experimental rooms

Experimental animals were ovigerous females of the terrestrial red-handed crab (akate-gani) *Sesarma haematocheir*, randomly collected on 9, 19, 31 July, 16 August 1990, 19 July, and 8 August 1991 from the thicket along a small estuary at Kasaoka, Okayama Prefecture. The crabs were immediately brought into the experimental rooms in the laboratory, and were kept in plastic containers (70 cm long, 40 cm wide, and 25 cm high) with shallow water (1 cm deep) at the bottom, and with hiding spaces above it. Light and temperature in the experimental rooms were controlled. A 15-h light: 9-h dark photoperiod, the same phase as that in the field (light-off at 20:00 and light-off at 5:00), was employed for all experiments. The intensity of illumination in the light phase was 700–1200 lux at the floor, and in the dark phase, less than 0.05 lux. Temperature was constant at 23 ± 1.5°C and the crabs were fed every few days.

A female of *S. haematocheir* incubates 20,000–50,000 eggs on her abdomen. When embryonic development is complete, all of the larvae hatch simultaneously. Hatching is completed within a very short time, within 5–30 min in the laboratory (Saigusa, 1992c). As soon as hatching is finished, the female releases her larvae into the water. The time of day of larval release can easily be monitored by the photoelectric-switch method (Saigusa, 1992a). Under the above-mentioned light conditions, larval release activity shows a circa-tidal rhythm, the phase of which coincides roughly with nocturnal high waters.

Exchange of egg clusters between two females

The females of *S. haematocheir* incubate their eggs for about one month. During this time, the color of the embryos changes from dark brown to brownish green, according to the stage of development which can, therefore, be estimated by visual inspection. In these experiments, females with mature embryos (brownish green color) or near mature embryos (light brown color) were used.

The reciprocal exchange of a cluster of embryos between paired females is carried out as follows. Two females with similar carapace sizes were taken from containers. The walking legs and body, except the portion where the embryos are incubated, were wrapped in a paper towel, and the claws were then secured with a rubber band (Fig. 1A, upper panel). To prevent the crabs from removing the

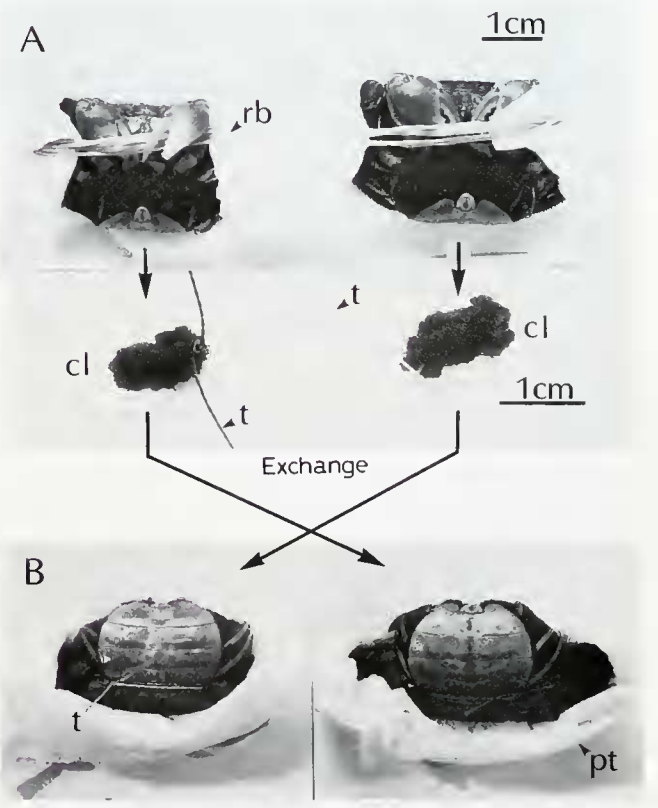

Figure 1. Embryo exchange between two females. (A) Upper photo: females with their chelae and walking legs restrained with a rubber band (*rb*). Lower photo: a cluster of embryos (*cl*) detached from each female. The base of the ovigerous seta is tied with a thread (*t*). (B) Females after the embryo exchange: a view from behind. *pt:* paper towel.

exchanged cluster, 2–3 mm of one of the (paired) tips of both claws was removed with scissors (see the female at the left side of Figure 1A). Bleeding was stopped with a small soldering iron.

Females of *S. haematocheir* have four pairs of abdominal appendages, each of them consisting of plumose and non-plumose setae (Fig. 2). The eggs are attached, just like grapes (8 clusters in all), to ovigerous hairs that grow from the non-plumose setae, and are ventilated by the female during development. The number of attached embryos in a cluster is 2000–6000. The first non-plumose seta on the right side was cut with scissors (Fig. 2), because it is the most convenient place to bind an exchanged cluster from another female. The excision of the egg cluster caused a small amount of bleeding from the base of the non-plumose seta, but hemostasis was induced with a sharpened soldering iron. These procedures, including the removal of an egg cluster, were applied in rapid succession to both females.

Each cluster of removed eggs was tied at the cut end of its seta to the center of a long thread (Fig. 1A, lower panel). Each tied cluster was then put into the space where the reciprocal cluster had been detached, and the free ends of the thread were passed around to the dorsal side of the abdomen and knotted at the articulation between the abdomen and the thorax (Fig. 1B). This prevented the transplanted cluster from being squeezed out of the egg mass being incubated by the host female. There was no exchange of blood between the transplanted non-plumose seta and the female.

Paralleling the exchange of a cluster of embryos, another small embryo cluster (200–500 eggs) was removed from each female and placed in the glass beaker with aeration (Saigusa, 1992b). Under such conditions, embryos that were detached less than 48–49.5 h before the larval release, all hatch on the same night as the eggs incubated by the female; moreover, they develop and are able to swim. In contrast, embryos separated earlier than 48–49.5 h before the release, do not hatch during the experimental period. After more than a week of aeration, these embryos gradually hatch as larvae with no ability to swim (*i.e.*, the prezoea) (Saigusa, 1992c). To determine whether the hatching of transplanted embryos is triggered by the host female, hatching of the embryos detached from the female was monitored (*i.e.*, control experiment).

The time required for the removal and exchange of a pair of egg clusters was about 15–20 min. In addition, preparation of the control experiment—*i.e.*, detachment of a small egg mass, binding it with thread, and then setting it onto the apparatus for aeration—took only about 5 min. To avoid nocturnal light, procedures were carried out in experimental rooms with a light phase of 24-h LD cycle.

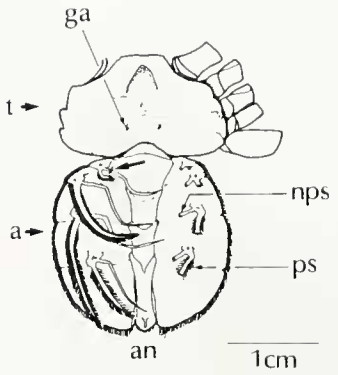

Figure 2. Abdominal appendages of *Sesarma haematocheir* female. The abdomen is opened and drawn from the ventral aspect. (*t.* thorax, *a.* abdomen, *an.* anus, *ps.* plumose seta, *nps.* non-plumose seta, *ga.* genital aperture). Ovigerous hairs growing from the non-plumose seta are omitted from the drawing. The unlabeled black arrow shows the place where the cluster of embryos is cut off with scissors.

Inspections of hatching in transplanted embryos

When the exchange of a cluster of embryos had been completed, the females were put into individual plastic cages with small holes in their sides. (These cages were either 11 cm in diameter and 10.5 cm in height, or 7 cm in diameter and 14 cm in height.) Each cage was then placed in a beaker containing 10‰ clean seawater. The time of larval release was monitored with a photoelectric device; details of this apparatus have already been described elsewhere (Saigusa, 1992a).

One of the most important questions in the present study was whether the transplanted cluster of embryos (Fig. 3A) would successfully hatch, and if so, whether this would occur simultaneously with the 7 clusters of female-attached eggs. For this purpose, hatching was also monitored, not only with the photoelectric apparatus, but also by visual inspections, described in detail below (Fig. 4A, B).

In intact females of *S. haematocheir* (*i.e.*, females without embryo exchange), hatching occurs synchronously, possibly within 5–30 min in the laboratory. Eggs were frequently found to be wet from the diluted seawater in the beaker due to the female's movements within the cage. When the hatching started, several zoea larvae were observed swimming in the beaker (Fig. 4A, middle). As soon as hatching was completed, the female released all the larvae into the water within 3–5 s (Fig. 4A, right). This quick release is associated with an abdominal fanning behavior, which triggers the photoelectric switch. Thus, if the seawater in the beaker is frequently checked, an obvious sign of hatching (*i.e.*, several swimming zoeas) will be noticed about 30 min before the larval release for most specimens. Such visual inspections were also applied to females with a transplanted cluster of embryos.

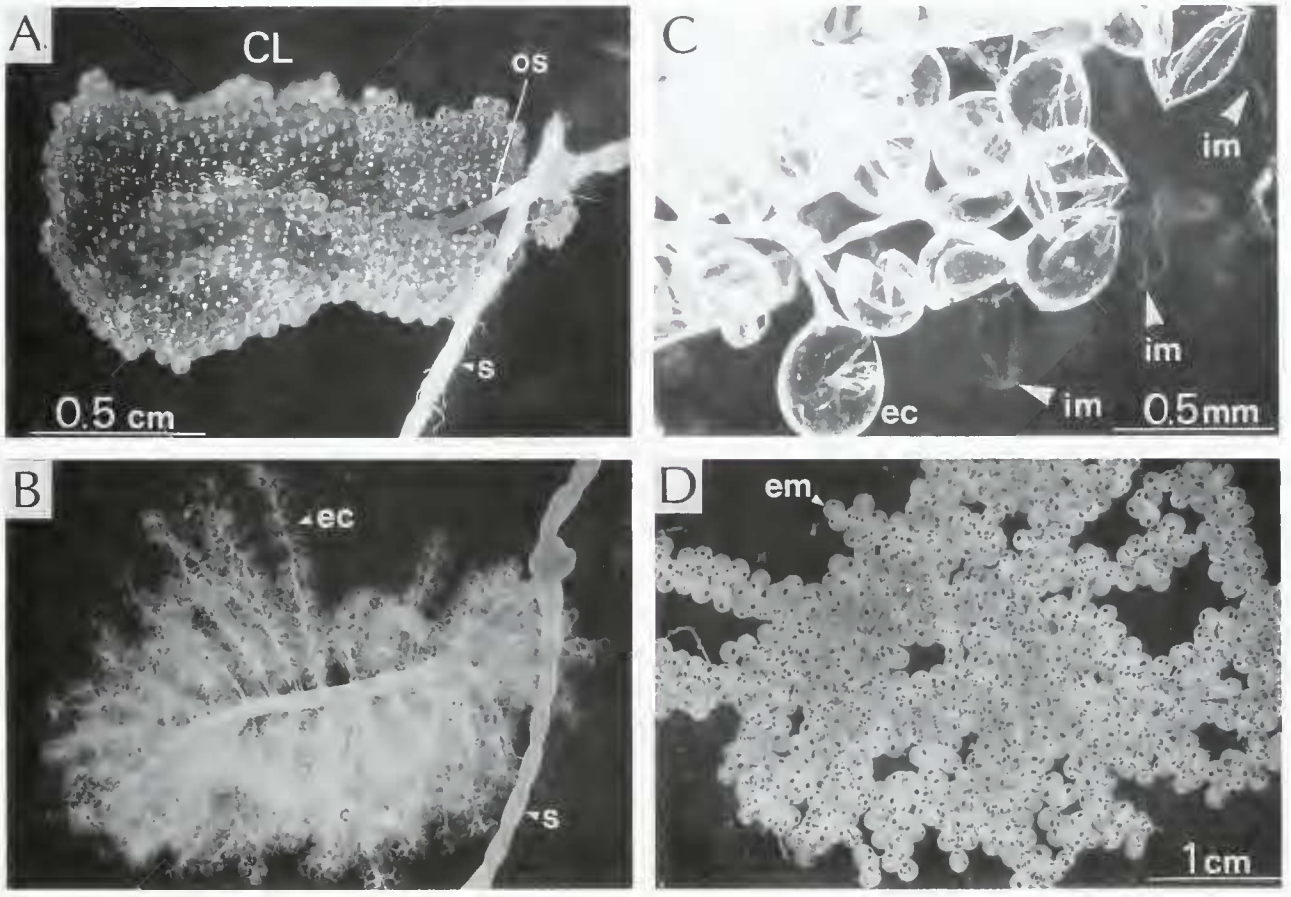

Figure 3. Eggs of *S. haematocheir* and their hatching. (A) a cluster of embryos (*CL*) the cut base of which is tied with a fine thread (*s*). *os*: ovigerous seta. (B) empty egg-cases (*ec*) remaining after larval release by the female. (C) very thin membranes (*im*) protruding from the egg-case upon the liberation of hatched larva. This membrane invests the embryo before hatching [described as the "third membrane" by Saigusa (1992b), but probably the cephalic portion of the so-called embryonic cuticle]. (D) embryos (*em*) dropped from ovigerous hairs without hatching. Diameter of each egg, about 330–350 μm.

Observations were made every 15–30 min throughout the night, using a hand-held light (or head lamp) covered with a few sheets of red cellophane. (These red lights were used for all of the observations and manipulations carried out in the experimental rooms during the dark phase.) When several zoeas were found swimming, the beaker was examined more frequently, *i.e.*, at intervals of 5–10 min.

As the upper diagram of Figure 4B indicates, when the photoelectric switch monitoring one of the paired females with a transplanted cluster operated (*i.e.*, the sign of larval release), the female was taken out of the cage. The thread was cut, and the transplanted cluster was carefully removed from the female's abdomen. Since empty egg cases remain attached to the ovigerous hairs, as they do after a normal larval release, it was easy to determine whether all of the transplanted embryos had hatched. (The judgment as to whether all the eggs hatched at the same time as the other embryos carried by the female is described in the Results section.) This observation was made under normal light, outside of the experimental room, because a female that had completed larval release was never used for further experiments. If hatching had not yet occurred, the cluster was quickly transferred into vigorously aerated seawater (10‰), and examined for the subsequent occurrence of hatching. Some of the aerated clusters were monitored for hatching every hour in constant darkness (DD) or in 24-h light-dark (LD) conditions; the hatching of other clusters was sought during the light phase of the 24-h LD cycle.

Just after the observations and manipulations mentioned above, the *other female* was also checked to examine whether her transplanted cluster had also hatched. This was done by observing the water in the beaker under red light. As indicated in the middle diagram of Figure 4B, when hundreds of zoeas were seen swimming in the beaker, the transplanted cluster was removed and its

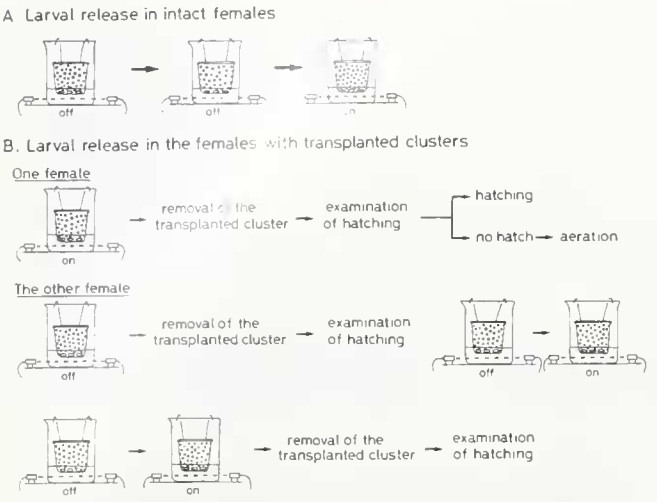

Figure 4. Experimental procedures used to examine hatching in intact females and females with transplanted clusters. The female crabs (not depicted) are in perforated cages suspended in beakers containing dilute seawater (10‰). A photoelectric device monitors the seawater for the presence of zoeas (see text). (A) The sign of hatching and larval release by intact females. Before larval release, the photoelectric switch is "off." Upon larval release, the switch operates ("on"); threshold of response: 10,000–20,000 zoeas in the beaker. (B) Examination of the transplanted clusters removed from host females. *Upper diagram:* Larval release occurs in *one* of the paired females, and the procedures indicated are followed to monitor hatching in the transplanted cluster (described in Materials and Methods section). *Middle diagram:* The cluster transplanted to the *other* female has hatched, indicated by hundreds of zoeas swimming in the beaker; (observed visually—the switch is "off"). The transplanted cluster is removed and examined, and the female is reset in the recording apparatus. *Lower diagram.* No zoeas are seen in the beaker. The female is removed from the cage *after* the release of her larvae, and the transplanted cluster is then examined.

hatching was confirmed; a stereo-microscope was used as necessary. The beaker was then replaced with another, and the female was reset to the recording apparatus. On the other hand, as shown in the lower diagram (Fig. 4B), there were many cases where no zoeas were seen swimming. In these cases, when the female finally released larvae, the transplanted cluster was removed and its hatching was examined.

Notice that the transplanted clusters were examined under normal light to determine whether hatching had occurred. This was no problem when all of the embryos had already hatched. But a question remained in the other cases as to whether this light might have affected the timing of hatching. To reduce the effect of light, some of the transplanted clusters were removed from the female in the experimental room under red light and transferred into a vigorously aerated medium. The latter experiments were carried out in 1991 as specified in the figures. The embryo exchange experiments involving 51 pairs of females were all done in 1990, although the year is not identified in the figures. Animals were never used for more than one experiment.

Results

Ovigerous females with a transplanted cluster released their zoea larvae between the night of embryo exchange (*e.g.*, Fig. 5A-a) and 11 days after. The release behavior was the same as that of intact animals. The results are clearly divided into the following three patterns (*Pattern I, Pattern II,* and *Pattern III*), which are related to the number of nights until larval release occurred.

Pattern I (Fig. 5A)

Both females released their larvae *within two nights* after the embryo exchange. The detached eggs of the control experiment and the transplanted embryos, all hatched on the same night that the *donor* females released their larvae. The results of this data can be further divided into three sub-patterns.

Sub-pattern I-1 (1 pair). As shown in Figure 5A-a, larval release of both females (F-1 and F-2) occurred on the night following embryo exchange. As soon as the release of one of the females (F-1) was recorded, the transplanted eggs (cl:F-2) were removed. Almost all eggs remained unhatched, and were quickly transferred to aerated conditions and monitored. As shown in Figure 5B-a, all of the embryos had hatched by about 6:00 on 18 August. On the other hand, the larval release of the paired female (F-2) occurred 50 min later than F-1 (Fig. 5A-a). When the implanted cluster (cl:F-1) was removed from F-2, it had already hatched. The eggs detached from both females (*i.e.*, ae:F-1 and ae:F-2) also hatched during the same night.

Sub-pattern I-2 (6 pairs). Larval release of these females occurred on the first and second nights after embryo exchange, respectively (Fig. 5A-b). The egg mass of the control experiment (ae:F-3 and ae:F-4) hatched on the same night that the donor females released their larvae. The transplanted eggs (cl:F-4) were removed from the female (F-3) immediately after larval release. No eggs hatched, and this cluster was monitored under constant darkness. Hatching occurred on the following night (Fig. 5B-b), corresponding to the release of the donor female (F-4). On the other hand, in the beaker where the female F-4 was confined, swimming zoeas emerged around the time of larval release of female F-3. These larvae had clearly hatched from the implanted cluster (cl:F-3). When this cluster was removed from the host female F-4, almost every embryo had already hatched. The remaining embryos hatched within a few hours under aerated conditions.

Sub-pattern I-3 (1 pair). In only one instance did both females release larvae two nights after the embryo exchange (Fig. 5A-c). The embryos in the control exper-

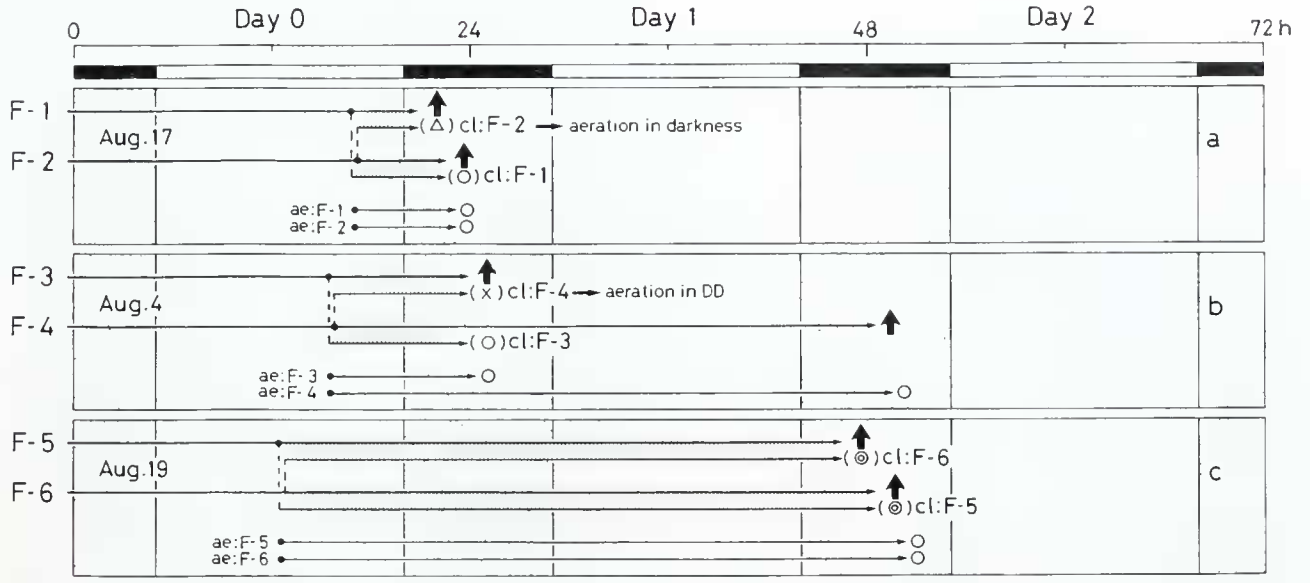

Figure 5A. Hatching of transplanted clusters: *Pattern I* (*a*) *Sub-pattern I-1* F-1 and F-2 designate the paired females that were set into recording apparatuses. Excision and exchange of embryos occurred at 16:55–17:15 on 17 August. Upward black arrows show the time of day of larval release of these females (at 23:00 for F-1, and at 23:50 for F-2). cl:F-2 and cl:F-1 are the transplanted clusters. ae:F-1 and ae:F-2 are the control egg masses kept in vigorous aeration. ○: Hatching of the egg-cluster or egg mass. △: Partial hatching of the egg cluster. (*b*) *Sub-pattern I-2*. Detachment and exchange of eggs, 15:20–15:40 on 4 August. F-3: Larval release at 1:00 on 5 August; F-4: at 1:40 on 6 August. ×: No eggs hatched from the transplanted cluster when it was removed from the host female. (*c*) *Sub-pattern I-3* Detachment and exchange of eggs: 12:55–13:10 on 19 August. F-5: Larval release at 23:50 on 20 August; F-6: at 0:50 on 21 August. ◎: These transplanted egg-clusters hatched at the same time as the female-attached eggs. Other symbols as in Fig. 5A-a and 5A-b.

iment (ae:F-5 and ae:F-6) both hatched on the second night after the embryo exchange. A feature of this case was that hatching of the transplanted cluster (cl:F-6) seemed to be *synchronized* with that of the host female (F-5). On the other hand, no swimming zoeas were observed in the beaker of the female F-6 around the time of the release of F-5, although the beaker was checked often. Female F-6 released her larvae before the transplanted cluster (cl:F-5) was removed. When this cluster was examined, all the egg cases were already empty (Fig. 3B). Thus, hatching of this transplanted cluster also seemed to be synchronized with that of the female-attached eggs.

Pattern II (Figs. 6A and 7A)

In this pattern, only one of the paired females released her larvae *within one or two nights* after the embryo exchange. The embryos of the control experiment all hatched on the same night as the release of the donor female. In contrast, the paired females released their larvae *more than three nights* after the embryo exchange. The control egg masses taken from these females did not hatch at all. A remarkable feature was that the hatching of the transplanted cluster was apparently induced by the donor female. The results were further divided into the following two sub-patterns.

Sub-pattern II-1 (11 pairs). The first pattern occurred when one of the females released larvae on the *first night* after embryo exchange. Five instances are summarized in Figure 6A. For example, in Figure 6A-a, larval release of F-7 occurred on the night of embryo exchange. The transplanted cluster of embryos (cl:F-8) was removed from the female 10 min after the release of F-7, but no eggs had yet hatched. This cluster was quickly transferred to aerated conditions, and was monitored every hour (Fig. 6B-a).

The reciprocal cluster (cl:F-7), which had been transplanted to the female F-8, hatched on the same night that the donor female (F-7) released her larvae. When the beaker of F-8 was observed with the red light shortly after the larval release of F-7, hundreds of zoea larvae were swimming. The transplanted cluster (cl:F-7) was quickly removed from F-8 under red light, and examined. The zoea larvae remaining attached to broken egg cases began to swim when the cluster was shaken by hand several times in the seawater, and hatching was completed within

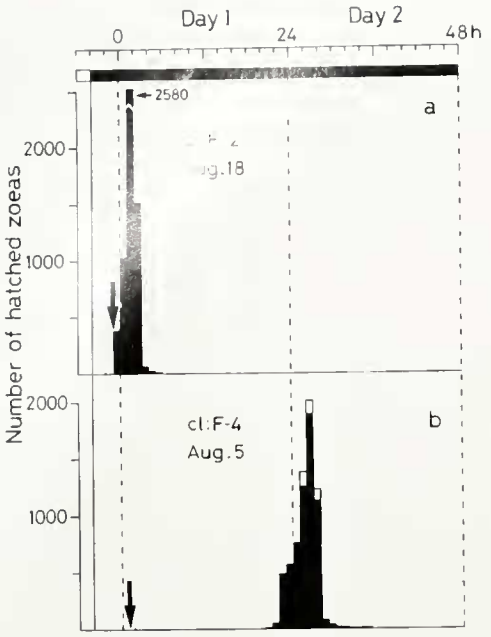

Figure 5B. Hatching of the transplanted egg-clusters. (*a*) Hatching of the cluster (cl:F-2) removed from the host female (F-1). Downward black arrow: time of larval release of the host female (F-1). (*b*) Hatching of the cluster (cl:F-4) removed from F-3. Open areas in histogram indicate the number of premature zoeas (prezoeas) that could not swim and thus sank to the bottom of the beaker.

a few hours. The beaker of F-8 was replaced with another one containing 10‰ seawater, and larval release of this female was monitored.

The control egg mass (ae:F-8) never hatched even after five days (Fig. 6A-a). In contrast, the cluster of embryos (cl:F-8) successfully hatched two nights after the start of aeration (Fig. 6B-a), and all eggs hatched by noon on 10 August. Obviously, hatching of the transplanted embryos (cl:F-8) was *induced* by the host female (F-2).

As four additional cases show (Fig. 6A-b-e), hatching of the transplanted clusters (cl:F-10, cl:F-12, cl:F-14, cl:F-16) was induced by the host females. Moreover, when these embryos were transferred into aerated conditions, they always hatched *two nights* after the removal from the host female (compare Fig. 6A and 6B). The pattern of hatching was similar, whether the clusters were monitored in constant darkness (Fig. 6B-a-b), or in LD cycles (Fig. 6B-c-e).

Sub-pattern II-2 (7 pairs). In these cases (Fig. 7A), the *first* larval release occurred on the *second night* after the embryo exchange. The control egg mass detached from these females hatched on the same night. For example (Fig. 7A-a), female F-17 released her larvae first. The cluster transplanted to this female (*i.e.*, cl:F-18) was quickly removed and examined with the stereo-microscope. No hatching had occurred, so this cluster was transferred into aerated conditions and monitored. After about 24 h, it had completely hatched (Fig. 7B-a).

At about the time that F-17 was releasing her larvae, zoeas were observed swimming in the beaker of female F-18 (Fig. 7A-a). The cluster transplanted to this female (cl:F-17) was removed and examined. Most egg cases were already empty and the remaining embryos all hatched within a few hours in aerated dilute seawater. Female F-18 was replaced in 10‰ seawater, and monitored until the time of larval release (day 3; Fig. 7A-a).

Other transplanted clusters put into aerated conditions (*i.e.*, cl:F-20, cl:F-22, cl:F-24, cl:F-26) also hatched about 24 h after the larval release of their host females. In the first three experiments (Fig. 7A-a-c), the transplanted clusters (*i.e.*, cl:F-18, cl:F-20, and cl:F-22) hatched on the same night as the larval release of the donor females (*i.e.*, F-18, F-20, F-22). But in the other two instances (*d* and *e*), the donor females (F-24 and F-26) released their larvae two or three nights later than their complementary pairs (F-23 and F-25). These results further indicate that hatching of the transplanted clusters is induced by the host female.

Pattern III (Fig. 8)

In these experiments (25 pairs), *both* females released their larvae *more than three nights* after the embryo exchange. In these cases, none of the control egg masses ever hatched during the experiment. Five instances of hatching by the transplanted clusters are summarized in Figure 8. For example, one of the females (F-29) released her larvae five nights after the embryo exchange (Fig. 8-b). Within a few minutes after the photoelectric switch had operated, the transplanted cluster was removed from female F-29 and examined resulting in the discovery that all the embryos had already hatched (Fig. 3B). The beaker containing the paired female (F-30) was frequently checked on the night that female F-29 released, but no swimming larvae were seen, and larval release occurred on the following night. Female F-30 was removed from the cage 5 min after the release, and the transplanted cluster was examined. Again, it was observed that hatching was complete. Similar results were obtained from the other experiments shown in Figure 8-a and 8-c-e.

The following evidence suggests that all of the transplanted clusters in *Pattern III* hatched simultaneously with the attached clusters that had been incubated by the donor female. (1) In intact females, a small number of zoea larvae begins to swim in the beaker around 20–30 min before the larval release. Animals with transplanted embryos exhibited the same phenomenon. (2) When the transplanted cluster of eggs was examined just after the larval release of the host female, hatching had already been complete. (3) The date of larval release for the complementary

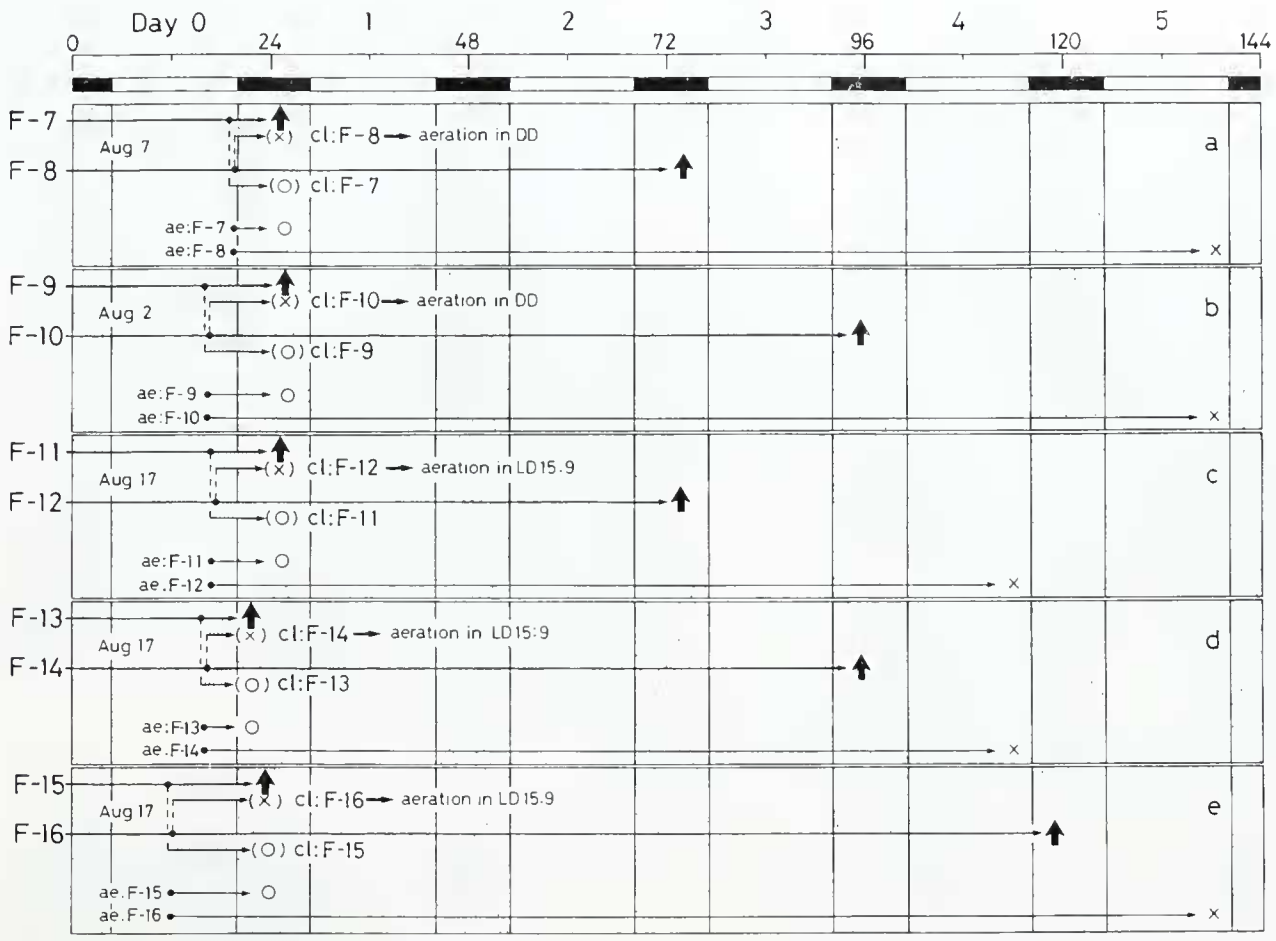

Figure 6A. Induction of hatching in the transplanted cluster of embryos: *Sub-pattern II-1*. (*a*) Embryo exchange between females F-7 and F-8. Detachment and exchange of clusters: 19:20–19:40 on 7 August. Larval release: F-7, at 1:00 on 8 August; F-8, at 2:00 on 10 August. (*b*) Embryo exchange between F-9 and F-10. Detachment and exchange of clusters: 15:50–16:20 on 2 August. Larval release: F-9, at 1:50 on 3 August; F-10, at 23:45 on 5 August. (*c*) Embryo exchange between F-11 and F-12 at 17:50–18:10 on 17 August. Larval release: F-11, at 1:00 on 18 August; F-12, at 1:25 on 20 August. (*d*) Embryo exchange between F-13 and F-14 at 16:10–16:30 on 17 August. Larval release: F-13, at 21:45 on 17 August; F-14, at 23:40 on 20 August. (*e*) Embryo exchange between F-15 and F-16 at 11:40–12:00 on 17 August. Larval release: F-15, at 23:40 on 17 August; F-16, at 22:40 on 21 August. The transplanted clusters (cl:F-7, cl:F-9, cl:F-11, cl:F-13, cl:F-15) all hatched on the same night as the larval release of the donor females. Symbols are the same as in Fig. 5A.

females was usually different. If these females carried their eggs for three nights or more after the embryo exchange, no swimming larvae appeared until the host female released her larvae. (4) When the transplanted cluster was examined with the stereo-microscope, a very thin membrane that invests the embryo had emerged from the egg case (Fig. 3C). Emergence of this membrane always accompanies hatching in this species (Saigusa, 1992b), so its appearance signifies that larvae had just emerged. Eventually this membrane is lost, and the inside of the empty egg cases are contaminated with fine detritus.

To prove that the transplanted clusters hatched simultaneously with the clusters attached to the host female, it was necessary to make direct observations of the female releasing larvae. Such observations were only meaningful if the females could be sampled within 30 min after the larval release was monitored in the event recorder (44 females). Shortly after larval release, the females unfold their abdomens and begin eating the empty egg cases that remain attached to the ovigerous hairs. Thus, six females that were examined more than 30 min after release had already eaten most of the empty egg cases, or the cases had dropped to the bottom of the beaker, so the time of hatching of either the transplanted or attached clusters could not be determined.

In *Pattern III*, there were only three instances in which

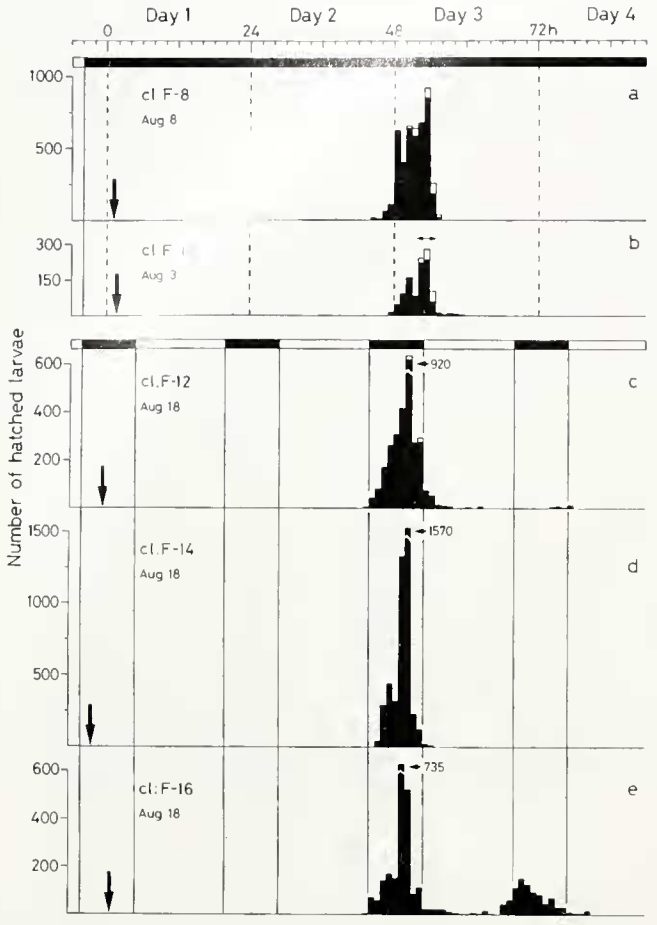

Figure 6B. Hatching of the transplanted egg-clusters in vigorous aeration. In the upper two panels (*a* and *b*), the cluster was monitored in constant darkness. In the lower three panels (*c*, *d*, and *e*), hatching was recorded in 24-h light-dark cycles. Note that the embryos hatch around 48 h after the larval release of the host females (downward black arrows). The horizontal arrow in panel *b* indicates that the egg masses dropped from cluster cl:F-10 during aeration, so the hatching was not fully monitored.

a portion of the transplanted cluster did not hatch, but remained attached to ovigerous hairs. These clusters were shaken by hand several times in 10‰ seawater to remove adherent zoeas, and were quickly transferred to continuously dark (DD) conditions and monitored under aerated conditions. These eggs all hatched with peak hatching occurring about 24 h later (not illustrated).

Loss of the egg sponge by the incubating female

Figure 9A indicates an example of egg loss, a curious phenomenon not usually seen in intact females, either in the laboratory or the field. In this experiment, one of the paired females (F-37) released her larvae on the night of embryo exchange. The egg cluster transplanted to this female (cl:F-38) was quickly removed and transferred into an aerated medium where it hatched about 48 h later. However, the reciprocally transplanted cluster (cl:F-37) was not removed quickly, therefore the egg sponge of the other female (F-38) dropped from her abdomen during the daytime of 2 August. At the time they were dropped, the embryos were still alive (Fig. 3D).

Thus, if a transplanted cluster *hatched* and was not removed soon thereafter, the host female's own attached eggs often dropped within a few days; indeed, 7 out of 52 females dropped all of their eggs without hatching. This phenomenon appears to be due to a substance released outside of the egg membrane and associated with hatching (Saigusa, submitted). Nevertheless, as shown in Figure 9A, the control egg mass (*i.e.*, ae:F-38) never hatched. So we can presume that female F-38 would have released larvae more than three nights after the embryo exchange. These results clearly belong to *Pattern II-1*. Eggs were lost only by females of *Pattern I* and *Pattern II*, and never in females of *Pattern III*. This is indirect evidence that, in *Pattern III*, transplanted embryos hatch on the same night as do the female-attached eggs.

Influence of the light used in monitoring on the hatching of the transplanted clusters

Hatching of transplanted embryos can be induced, if they are incubated by a host female that releases her larvae within two nights (Figs. 6B, 7B). Indeed, all of the transplanted clusters were removed from the host female under red light in the experimental chamber. But the determination of hatching was carried out under the normal light outside of the experimental room, although the time required for this observation was only 5 min. Hence, we must question the influence of this light on the hatching of these embryos.

To address this issue, the hatching profile of egg clusters exposed to normal light was compared with the profile of those exposed to red light. In this experiment, the transplanted cluster was removed from the host female just after larval release, and was kept under the light for 5 min. This cluster was then transferred into aerated conditions and was monitored every hour. In two other experiments, the transplanted clusters were also quickly removed from the host females just after larval release, but they were not exposed to the light outside of the experimental room. Instead they were tied to nylon thread under *red light* and monitored for hatching.

The hatching profiles of the cluster treated with normal light showed that hatching occurred two nights after the transfer into aeration (data not shown). The other two clusters, which had been treated with red light, also hatched two nights after the aeration. Furthermore, a small peak of hatching was also observed on the third night for

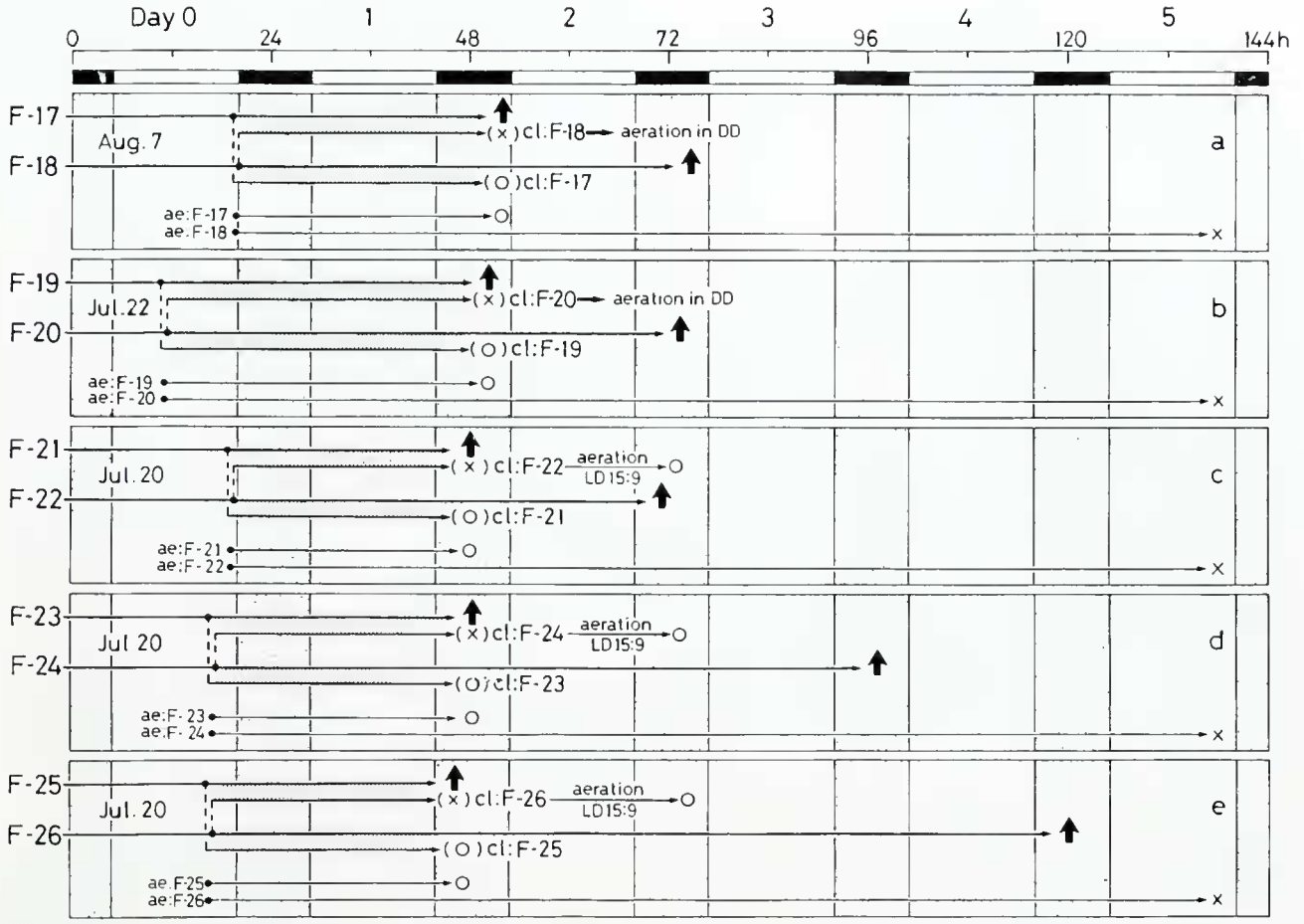

Figure 7A. Induction of hatching in the transplanted cluster of embryos: *Sub-pattern II-2*. (*a*) Embryo exchange between F-17 and F-18 at 19:45–20:00 on 7 August. Larval release: F-17, at 4:00 on 9 August; F-18, at 2:40 on 10 August. (*b*) Embryo exchange between F-19 and F-20 at 11:30–12:00 on 22 July. Larval release: F-19, at 2:30 on 24 July; F-20, at 1:30 on 25 July. (*c*) Embryo exchange between F-21 and F-22 at 18:30–19:00 on 20 July. Larval release: F-21, at 0:10 on 22 July; F-22, at 23:20 on 22 July. (*d*) Embryo exchange between F-23 and F-24 at 16:30–17:00 on 20 July. Larval release: F-23, at 0:20 on 22 July; F-24, at 1:10 on 24 July. (*e*) Embryo exchange between F-25 and F-26 at 17:10–17:40 on 20 July. Larval release: F-25, at 22:30 on 21 July; F-26, at 23:50 on 24 July. For hatching of egg-clusters (cl:F-18, cl:F-20) after they were removed, see Figure 7B-a and 7B-b. The other transplanted clusters (cl:F-22, cl:F-24, cl:F-26) hatched during one night later (hourly data not obtained).

one of these clusters. Such a secondary peak of hatching was often observed in other experiments treated with normal light (*e.g.*, Fig. 6B-e), so it cannot be attributed to the influence of red light (data not shown).

Some experiments related to the induction of hatching by the female

In two instances, the implanted egg-cluster did not hatch at all. Although egg loss occurred in both experiments, these results clearly belong to *Pattern II-1* (see Fig. 9B for the result of one of these experiments). A feature common to these two experiments is that the interval between the embryo exchange and the larval release of the host female was very short. The transplanted clusters were incubated by the host females for 4 h and 4.5 h, respectively. In every case of induced hatching, the minimum period was 5.5 h (*e.g.*, Fig. 6A-d). These results suggest that at least 5–6 hours are required to induce hatching of the transplanted cluster.

The possibility that some stimulus of hatching in the female-attached eggs induced the hatching of transplanted clusters was also examined. For this purpose, the transplanted cluster was removed from the host female some hours before the larval release (Fig. 9C). In this experiment, the clusters were incubated by the host females for about 17 h, and were then transferred into aeration. The cluster (cl:F-41) hatched on the same night that the donor

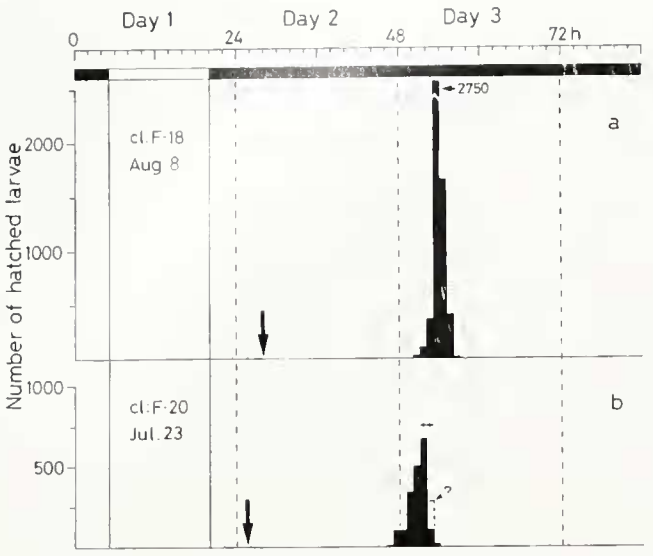

Figure 7B. Distribution of hatching in the transplanted embryos in vigorous aeration: (*a*) cl:F-18; (*b*) cl:F-20. The release of larvae by the host females (F-17, F-19) is shown by a downward arrow. (Egg masses dropped from the cluster at the time indicated by the horizontal arrow, and hatching could not be perfectly monitored during this period.) The other transplanted clusters from Figure 7A (cl:F-22, cl:F-24, cl:F-26) hatched one night later (hourly data not obtained).

female (F-41) released their larvae. The cluster (cl:F-42) also hatched two nights after the aeration. Since no hatching was observed in the control experiment (ae:F-42), hatching of cl:F-42 can be regarded as having been induced by the host female (F-41).

The possibility that egg-clusters kept under aerated conditions for more than three nights after detachment may lose their ability to hatch was then tested. In Figure 9D, a cluster (cl:F-43) was detached from a female and placed in aeration for five days. This cluster was then transplanted to another female (F-44). This cluster hatched in synchrony with the attached eggs of the host female F-44. Similar results were obtained in another experiment (not illustrated). Thus, eggs that were detached and transferred into aeration retained their ability to hatch. Hatching was obviously inhibited under aerated conditions.

The interaction between the female and transplanted embryos was also examined. A few clusters were covered with a skirt made of thin cellophane. This skirt was open at the bottom, ensuring an interchange of water at the surface of the eggs. As seen in Figure 10, the transplanted clusters were induced to hatch (upper left in each panel). But the hatching profiles of the egg-clusters that were transferred into aeration (Fig. 10) showed a somewhat different pattern. In these three experiments, the cluster hatched in two peaks, about 24 h apart. Almost all of the eggs hatched in one experiment (Fig. 10-a), but many eggs failed to hatch in the other two (Fig. 10-b-c). These results are difficult to interpret; one possible explanation is that the stimuli recognized by the embryos are attenuated by the cellophane, which caused the occurrence of two peaks and the decrease of the number of larvae hatched. In any event, such a splitting of the hatching pattern has never been observed in intact females.

Does the hatching of transplanted clusters affect the day or the time of hatching of eggs attached to the host female? For example, the hatching of the transplanted cluster (Figs. 5A, 6A) might release a stimulus that acts on the female to disturb the time of hatching or to advance the day of hatching of the female-attached eggs. To examine the former possibility, the time of day of larval release by host females (except the females whose eggs had dropped) was monitored with the event recorder. As shown in Figure 11, the larval release of these females coincided roughly with the time of night high water in the field, showing a clear circa-tidal rhythm. This suggests that at least the daily timing of egg hatching was not disturbed, either by the exchange of embryos or by the hatching of the transplanted eggs.

To examine whether the hatching of transplanted egg-clusters advances the date of hatching in the host female, the number of nights between the larval release of paired females was compared with respect to differences between *Pattern II* and *Pattern III*. The range was 0–9 days in *Pattern III* (25 pairs), but only 0–4 days in *Pattern II* (19 pairs with no egg loss). Since the experimental crabs were chosen randomly, this difference might suggest that hatching of the transplanted cluster can advance the day of hatching in the host female.

Discussion

The embryos of most marine crustaceans are incubated by the female for a certain period before hatching. An endogenous factor has been suggested as operating in the hatching rhythms of many kinds of marine animals (Saigusa, 1992c). But does the endogenous component controlling rhythmicity occur within the embryo, its mother, or both? To answer this question, larval hatching must be examined, not only in the embryo, but also in the female. Although the embryos of crustaceans are attached to non-plumose setae by a funiculus that is possibly composed of chorion, there is no circulation of blood between embryo and mother (Yonge, 1937, 1946; Cheung, 1966; Goudeau and Lachaise, 1983). Thus, the embryo exchange experiments were aimed at revealing the site of the endogenous clock.

An endogenous clock times hatching in each embryo

The present experiments were primarily aimed at determining whether the implanted embryos would hatch

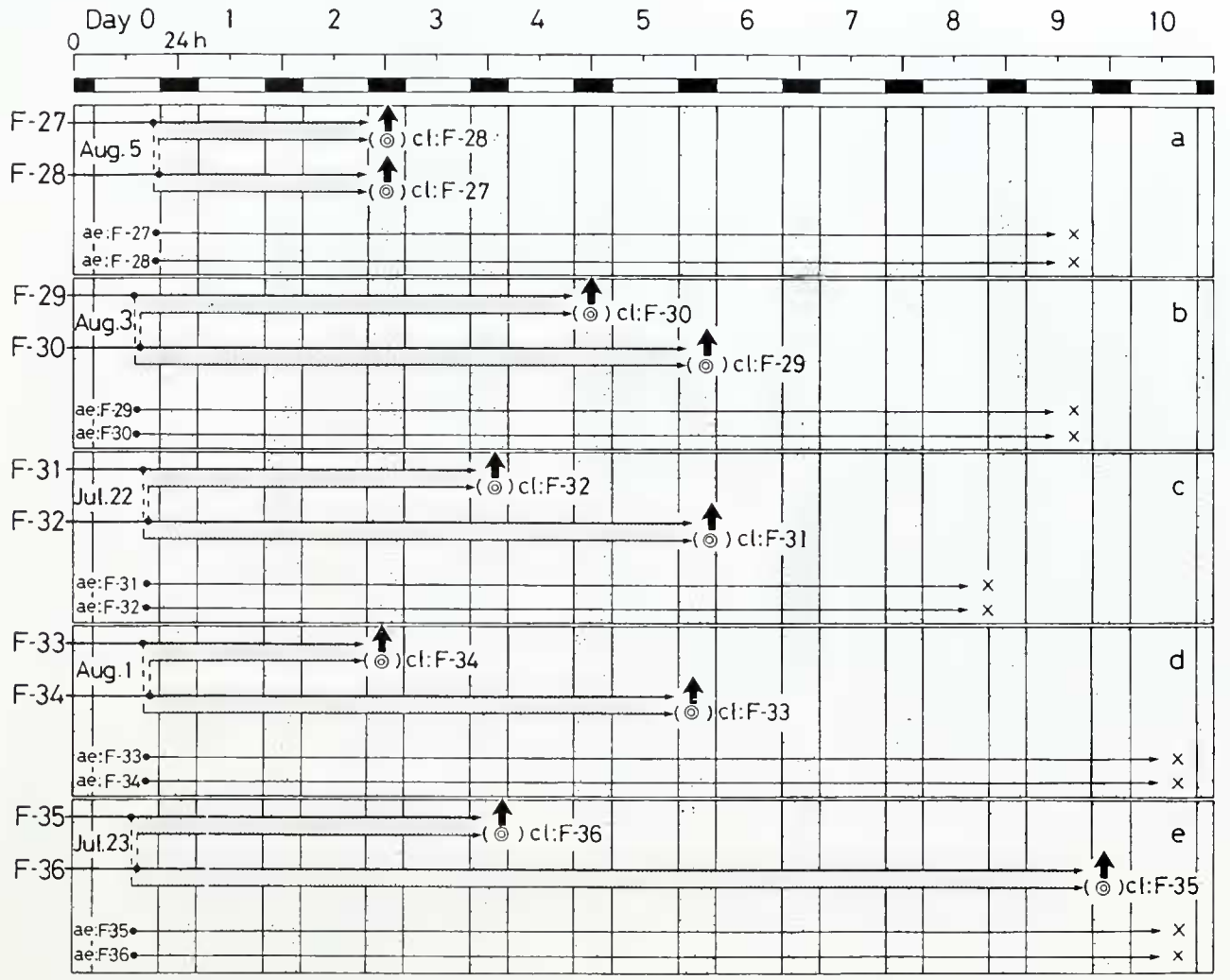

Figure 8. Induction of hatching in the transplanted cluster of embryos; *Pattern III* (*a*) Embryo exchange between females F-27 and F-28. Detachment and exchange of clusters: 19:20–19:40 on 5 August. Larval release: F-27, at 0:30 on 8 August; F-28, at 0:40 on 8 August. (*b*) Embryo exchange between F-29 and F-30 at 14:45–15:10 on 3 August. Larval release: F-29, at 0:35 on 8 August; F-30, at 3:05 on 9 August. (*c*) Embryo exchange between F-31 and F-32 at 16:30–17:00 on 22 July. Larval release: F-31, at 2:10 on 26 July; F-32, at 3:20 on 28 July. (*d*) Embryo exchange between F-33 and F-34 at 16:25–16:45 on 1 August. Larval release: F-33, at 23:35 on 3 August; F-34, at 23:50 on 6 August. (*e*) Embryo exchange between F-35 and F-36 at 14:15–14:40 on 23 July. Larval release: F-35, at 3:40 on 27 July; F-36, at 22:40 on 1 August. Symbols the same as in Figure 5A.

synchronously with the attached embryos of the host female. A potential procedural problem remaining is that the transplanted embryos were exposed to light, although for only 5 min, to determine whether hatching had occurred. Hatching could have been induced by the direct influence of this light, *e.g.*, like the oviposition of the teleost *Oryzias* (Egami, 1954; Ueda and Oishi, 1982). To reduce the effect of light, transplanted clusters were removed from the female, and subsequent procedures involving aeration were carried out under red light. The hatching pattern under red light was similar to that of an egg-cluster exposed to normal light. Furthermore, the peak of hatching occurred about 24 or 48 h after the transplanted egg-clusters had been transferred into aeration, even in constant darkness (Figs. 5B-b, 6B-a-b, 7B-a-b). These peaks must have been induced by an *endogenous* rhythm existed in embryo itself, and not caused by the light used during examination of hatching or by 24-h LD cycles.

Like hatching, pupation, and emergence, many aspects of development occur only once in the life cycle of an animal (Saunders, 1976). Whether the timing of those phenomena is controlled by an endogenous pacemaker can be examined in a population of mixed age *e.g.*, the

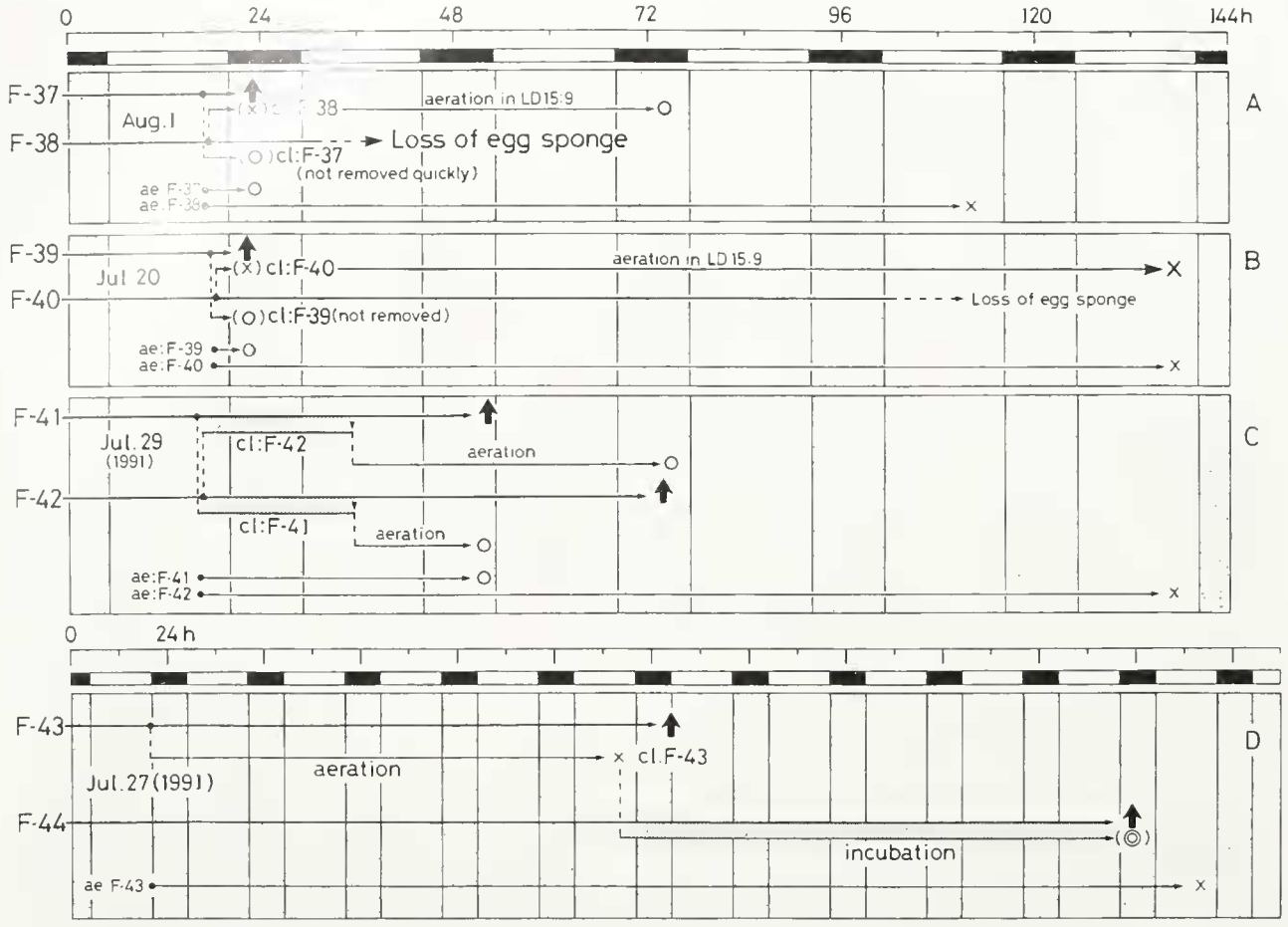

Figure 9. Hatching profile of transplanted clusters. (*A*) Loss of the egg sponge by the host female. Embryo exchange between females F-37 and F-38 at 16:10–16:25 on 1 August. Larval release: F-37, at 23:35 on 1 August. (*B*) Failure to induce hatching. Embryo exchange between females F-39 and F-40 at 17:50–18:20 on 20 July. Larval release: F-39, at 22:30 on 20 July. (*C*) Removal of the transplanted cluster before the release of the host female. Embryo exchange between females F-41 and F-42 at 16:05–16:25 on 29 July. Larval release: F-41, at 3:45 on 31 July; F-42, at 1:35 on 1 August. (*D*) Hatching of the egg-cluster kept in aeration for five days. Detachment of cl:F-43, at 19:50 on 27 July; binding to F-44, at 16:20 on 1 August. Larval release: F-43, at 4:55 on 2 August; F-44, at 23:10 on 6 August.

circadian rhythm of emergence in the fly *Drosophila* (Pittendrigh and Bruce, 1959). But the validity of using such a population to demonstrate a circadian rhythm has been questioned (Saunders, 1976, chapter 3). So Pittendrigh and Skopik (1970) used populations that were developmentally synchronous at pupation to study the emergence rhythm in the fly *Drosophila pseudoobuscura*, and suggested that a circadian pacemaker in each developing fly dictates the circadian time of *emergence*, but not that of the intermediate developmental stages, such as head eversion and eye pigmentation.

The eggs of *Sesarma haematocheir* are oviposited within a short time. So the developmental embryos incubated by a female clearly do not constitute a mixed-age population. Like the data presented by Pittendrigh and Skopik (1970), the hatching of the egg clusters removed from the host females was often split into two distinct peaks almost 24 h apart (Figs. 6b-e, 10a-c). To explain such a splitting of hatching pattern, we can assume an *allowed* zone (see Pittendrigh and Skopik, 1970) related to hatching in the endogenous pacemaker of each embryo. In Figure 12, this zone is expressed by the acrophase (shown by a dot) in the embryo's pacemaker. The preceding paper (Saigusa, 1992c) indicated that each embryo undergoes a hatching process that continues for 48–49.5 h prior to egg-membrane breakage. If the embryos were detached from the female early enough that this time interval were exceeded, hatching would not occur. One speculation is that, in the larval hatching rhythm in *Sesarma*, a gated phenomenon occurs at the start of the

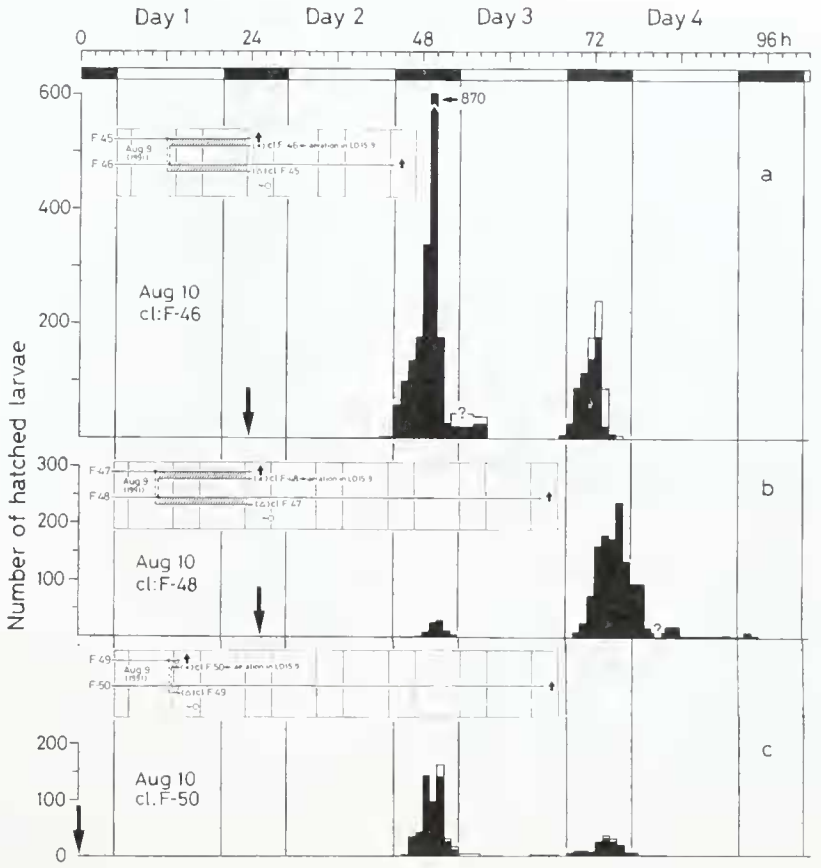

Figure 10. Hatching of egg-clusters from which the cellophane skirt was removed before transfer into aeration. (*a*) Hatching of cl:F-46. Embryo exchange between F-45 and F-46 at 17:25–17:40 on 9 August. Larval release: F-45, at 23:45 on 10 August; F-46, at 23:50 on 12 August. (*b*) Hatching of cl:F-48. Embryo exchange between F-47 and F-48 at 14:25–14:35 on 9 August. Larval release: F-47, at 1:30 on 11 August; F-48, at 1:50 on 15 August. (*c*) Hatching of cl:F-50. Embryo exchange between F-49 and F-50 at 19:10–19:20 on 9 August. Larval release: F-49, at 0:20 on 10 August; F-50, at 2:20 on 15 August. Downward arrows indicate time of day of release by the host females.

hatching process, and not hatching itself. If the hatching process is not initiated at a certain acrophase (Fig. 12), the embryos must wait until the next allowed zone to start the process.

Induction of hatching in the transplanted cluster

While each embryo has an endogenous rhythm of hatching, the present study indicates that the hatching of exchanged clusters is *induced* by the host female, provided that the incubation was longer than 5–6 h (compare Figs. 6A, 7A and 9B). Since the start of the hatching process may be a gated event, it is reasonable to speculate that this process begins 48–49.5 h before the hatching, responding to some signal to each embryo. But we do not know *what* stimuli trigger the hatching process in each embryo. One possibility is mechanical stimuli generated by the female—perhaps some special movements of the abdomen or ovigerous setae as the embryonic development is completed. Another possibility is a hatch-inducing substance, produced by the female and recognized by the embryos. We also do not know *when* hatch-inducing stimuli are released from the female. Females could generate such stimuli at any time of day, or at a particular phase of her circatidal rhythm (see the question mark on the female pacemaker in Fig. 12).

Synchronization of hatching between transplanted embryos and female-attached embryos

For most intertidal and estuarine crustaceans, female-attached eggs hatch within a very short period, although the exact duration cannot be determined because of the mass. In *S. haematocheir*, hatching is completed in 5–30 min in each female (Saigusa, 1992b). Because the hatching synchrony of the embryos detached from the female is perturbed (Saigusa, 1992c), some mechanism must underlie the highly-synchronous hatching in female-attached eggs. This cannot be an endogenous clock in each embryo;

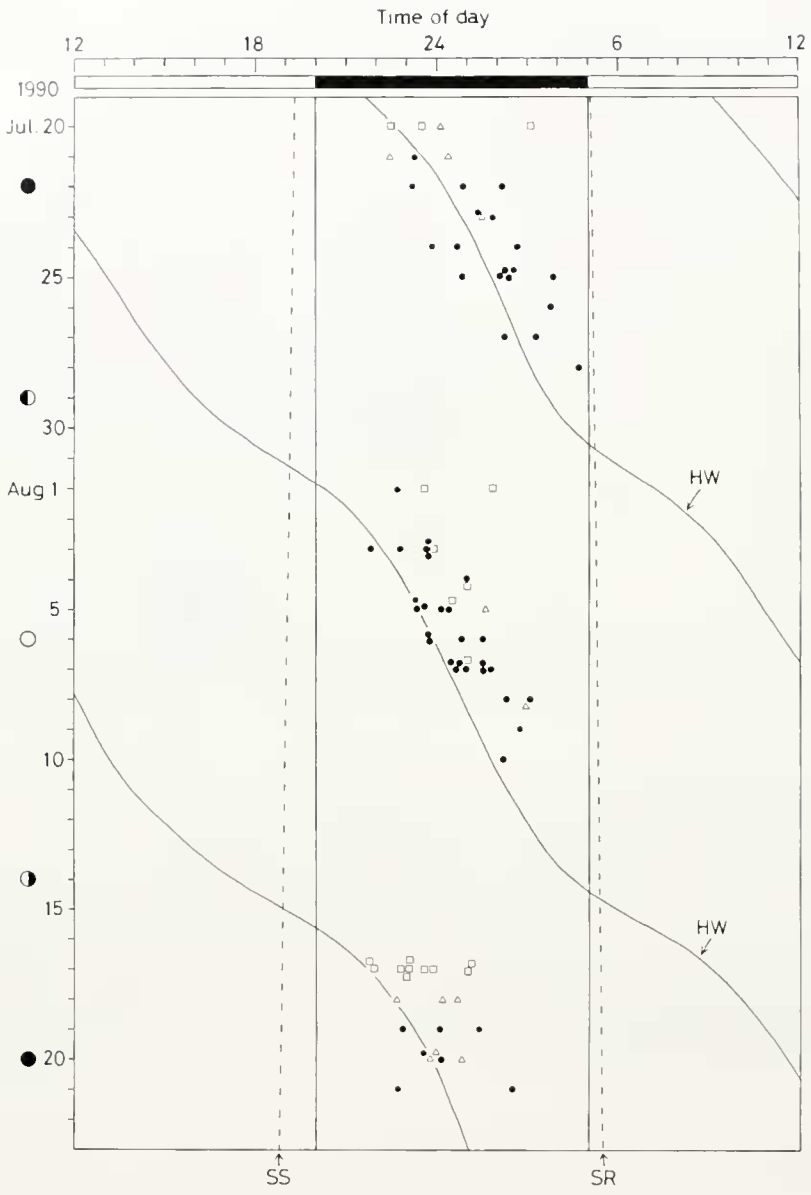

Figure 11. Time of day of larval release by females carrying an exchanged cluster and by females from which the cluster had been removed. Records were made in the laboratory under 24-h LD cycle, but under no tidal influence. Solid diagonal lines connect the times of high water (*HW*) in the field. □: larval release on the first night of embryo exchange. △: larval release on the second night after embryo exchange. ●: larval release occurs more than three nights after the embryo exchange. *ss* and *sr* connect the times of sunset and sunrise, respectively.

the female must produce some unknown stimulus that enhances synchronous hatching.

Females of *S. haematocheir* release their larvae with vigorous abdominal movements. This same behavior is observed in other terrestrial crabs (Saigusa, 1981). In species that release their larvae under the water, the release is effected by the pumping behavior of the abdomen (DeCoursey, 1979; Forward *et al.*, 1982; Saigusa, 1992a). Forward and Lohmann (1983) suggested that this behavior enhances hatching synchrony. In contrast, hatching in terrestrial species occurs prior to larval release. Clearly, larval release behavior itself does not enhance the hatching synchrony in female-attached embryos. One possible mechanism is that the female kneads the egg-clusters several times around the time of night high tide. In addition to such physical stimuli, the hatching of a few embryos might release a substance like the hatching enzyme suggested by De Vries and Forward (1991), and thus stimulate the hatching of the remaining embryos.

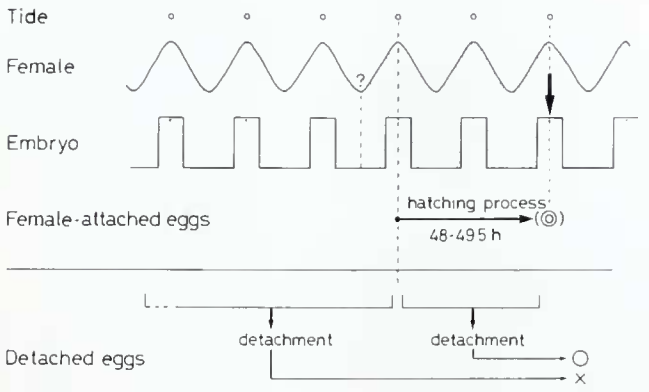

Figure 12. Proposed mechanism of induction of hatching and synchronization of hatching with nocturnal high water. Endogenous pacemakers related to hatching are shown with by a sine curve (female) and a rectangle (embryo). Small circle above the female's pacemaker indicates time of nocturnal high water. Stippled area: stimuli that induce the hatching process in each embryo are released during this period. The heavy downward arrow represents stimuli by the female to enhance hatching synchrony among embryos. The success of hatching in detached embryos is shown under the horizontal line. See the text for details.

Timing mechanism of hatching: a hypothesis (Fig. 12)

As in most other decapods, the oviposited eggs of *S. haematocheir* are incubated by the female until hatching occurs. Most of this period is probably related to embryonic development. But hatching does not immediately follow the completion of development; *i.e.*, embryos wait for stimuli that initiate the hatching process. So when the eggs are detached from the female during this period, hatching should be inhibited. As demonstrated in this study, one or more hatch-inducing stimuli are produced by the female. But once these signals have been received, the start of the hatching process would be determined by an endogenous clock within each embryo.

We can assume that a self-sustained oscillation underlies most endogenous rhythms (Pittendrigh and Bruce, 1959). As shown in Figure 12, we can express the embryo's pacemaker for hatching as a rectangular wave with 24.5-h period. Similarly, females would also have 24.5-h pacemaker for hatching and larval release. Both pacemakers would be synchronized with nocturnal high tide in the field. Since eggs that are detached from the female more than 48–49.5 h before hatching of the female-attached eggs do not hatch, the "allowed zone" related to the start of this process should be positioned at the phase corresponding to the time of nocturnal high tide, *i.e.*, the acrophase of the embryo's pacemaker in Figure 12. When some (unknown) stimuli (stippled area in Fig. 12) have been transmitted to the embryos for several hours (at least more than 5–6 h), the hatching process starts at this phase, and zoeas hatch 48–49.5 h later; *i.e.*, around the time of nocturnal high tide. If the stimuli from the female are insufficient to start the hatching process, embryos must wait for the next acrophase. This would have resulted in the split hatching peaks seen in vigorously aerated dilute seawater (Figs. 6B-e and 10 a-c).

As shown in the lower diagram of Figure 12, if the embryos are detached from a female before the hatching process, they would not hatch at all. If they are separated from a female while the hatching process is in progress, then those embryos will hatch at about the same time as the embryos that remain attached to the female. But hatching synchrony is perturbed in this condition, so the process is extended by several hours (Saigusa, 1992c). Since female-attached eggs hatch synchronously, the females must have some mechanism (a downward arrow in Fig. 12) for enhancing hatching synchrony while they are still on the hillside awaiting the time of hatching.

Acknowledgments

The zoea larvae found after hatching were counted by Mr. A. Shiomi and Miss H. Yunoki, students of Okayama University. They also helped with some of the daytime procedures, such as exchanging clusters, placing females in the recording apparatuses, and collecting ovigerous females. Supported by a Grant-in-Aid for Scientific Research (C) (No. 02640582) from the Ministry of Education, Science and Culture.

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